Probiotic yogurts manufactured with increased glucose oxidase levels: Postacidification, proteolytic patterns, survival of probiotic microorganisms, production of organic acid and aroma compounds

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ABSTRACT

We investigated the effect of increased glucose oxidase concentration as a technological option to decrease oxidative stress during the processing of probiotic yogurts. Probiotic yogurts were produced with increased concentrations of glucose oxidase (0, 250, 500, 750, or 1,000 mg/kg) and submitted to physicochemical and microbiological analysis at 1, 15, and 30 d of refrigerated storage. Higher concentrations of glucose oxidase (750 and 1,000 mg/kg) and a longer storage time were found to have an influence on the characteristics of the probiotic yogurt, contributing to more extensive postacidification, an increase in the dissolved oxygen level, and higher proteolysis. In addition, increased production of aroma compounds (diacetyl and acetaldehyde) and organic acids (mainly lactic acid) and a decrease in the probiotic bacteria count were reported. The use of glucose oxidase was a feasible option to minimize oxidative stress in probiotic yogurts. However, supplementation with excessive amounts of the enzyme may be ineffective, because insufficient substrate (glucose) is present for its action. Consumer tests should be performed to evaluate changes in the sensory attributes of the probiotic yogurts with increased supplementation of glucose oxidase. In addition, packaging systems with different permeability to oxygen should be evaluated.

Key words: glucose oxidase, probiotic yogurt, oxidative stress

INTRODUCTION

The consumer preference for yogurt as a vehicle for probiotic culture is much more related to the reputation

of a naturally healthy product in the consumer's mind than to the technological obstacles involved in its production (Zhu et al., 2009; Johansen et al., 2010; Sanders and Marco, 2010). Considering the food matrices that may be supplemented with probiotic microorganisms, such as soy, meat, cereal and fruit juices (Granato et al., 2010a; Rivera-Espinoza and Gallardo-Navarro, 2010), fruit (Alegre et al., 2011), cheeses (Bergamini et al., 2009; Albenzio et al., 2010; Gomes et al., 2011; Madureira et al., 2011a,b; Minervini et al., 2012) or ice creams (Di Criscio et al., 2010; Isik et al., 2011; Ferraz et al., 2012), yogurts and fermented milks are the most popular food carriers (Almeida et al., 2008; Oliveira et al., 2009, 2011; Allgeyer et al., 2010; Cruz et al., 2010b, 2012; Mortazavian et al., 2010; Marafon et al., 2011), and it is widely known that the consumption of probiotic products may confer many health benefits (Ejtahed et al., 2011; Ramchandran and Shah, 2011; Savard et al., 2011). Recently, Saxelin et al. (2010) demonstrated that yogurts and fermented milks were as effective as capsules for the administration of probiotic bacteria, emphasizing the importance of such matrices as functional food matrices.

Obtaining desirable therapeutic effects in probiotic dairy products such as yogurts and fermented milks requires the viability of the probiotic bacteria to be maintained at a sufficient level throughout storage of the product. It has been suggested that probiotics should be present in the food product in minimal amounts of 10^6 cfu/g, representing a daily dose of 10^8 cfu/g, to compensate for a possible reduction in this number during passage through the gastrointestinal tract (Granato et al., 2010a,b; Figueroa-González et al., 2011). From a technological standpoint, the processing of yogurt supplemented with probiotic cultures is not easy, particularly with respect to maintaining the viability of the cultures (Corcoran et al., 2008). One problem is that of oxidative stress, which is caused by exposure to oxygen permeating through the plastic packaging

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material during refrigerated storage (Granato et al., 2010a). To overcome the problem of oxidative stress, technological alternatives have been proposed. These include the addition of chemical compounds aimed at achieving a chemical solution called the destressing effect (Zhao and Li, 2008); the addition of *Lactococcus lactis* to milk intended to be used in the manufacture of yogurt to reduce its oxi-reduction potential (Jeanson et al., 2009; Yonezawa et al., 2010; Odamaki et al., 2011); the addition of nitrogen simultaneously with the addition of cultures during yogurt processing, followed by fermentation at 37°C (Horiuchi et al., 2009); the addition of gases to modify the redox potential (Ebel et al., 2011); and even the development of oxygen-tolerant strains (Li et al., 2010). Indeed, maintaining the viability of probiotic strains in dairy foods is still a challenge for the industry once many metabolic conditions (e.g., sensitivity to oxygen) need to be taken into account in order to develop a functional food that exerts a therapeutic function. Immobilization on different polymers (Rodrigues et al., 2011), manipulation of incubation temperature, final pH of fermentation (Shafiee et al., 2010), and even music waves (Mohammadi et al., 2011) are recent approaches to improve the viability of probiotic bacteria in probiotic fermented milks and yogurts.

The use of a glucose–glucose oxidase enzyme complex has been proposed as a viable alternative to reduce the oxygen dissolved in the product and consequently maintain *Bifidobacterium longum* counts at levels capable of delivering benefits to the consumer. Using this approach and surface response methodology, predictive models were obtained and validated, and glucose oxidase was found to be the variable of greatest importance for predicting *B. longum* counts (Cruz et al., 2010a), suggesting that the addition of increasing levels of the enzyme may have a positive effect on the probiotic status of the product. This research aimed to evaluate the effect of increased levels of glucose oxidase in the processing of probiotic yogurts. Postacidification, proteolytic patterns, survival of probiotic bacteria, and the production of organic acid and aroma compounds were evaluated during refrigerated storage.

MATERIALS AND METHODS

Culture Growth

Pure cultures of *Streptococcus thermophilus* TA040, *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340, *Bifidobacterium longum* BL05, and *Lactobacillus acidophilus* La14 were obtained from Danisco (São Paulo, Brazil) and maintained at –80°C. The *B. longum* strain was chosen due its higher sensitivity to oxygen compared with other *Bifidobacterium* strains (Simpson et al.,

2005; Kawasaki et al., 2006; Jayamane and Adams, 2009; Fortin et al., 2010).

Skim milk powder (Molico, São Paulo, Brazil), reconstituted to 11% (wt/vol), was used to prepare the lactic and probiotic cultures. For *B. longum* BL05, 0.05% (wt/vol) cysteine (Sigma, São Paulo, Brazil) and 0.5% (wt/vol) yeast extract (Oxoid, São Paulo, Brazil) were added to the milk powder reconstituted to 11%, whereas for the other lactic and probiotic cultures, the reconstituted skim milk was supplemented with 2% (wt/vol) glucose (Synth, São Paulo, Brazil). All the reconstituted milks were autoclaved at 115°C for 10 min, and the cysteine solution used was sterilized by membrane filtration.

Yogurt Processing

The probiotic stirred yogurts were produced using a standard yogurt manufacturing process (Tamime and Robinson, 2007), the operational parameters such as fermentation temperature being tested previously (Cruz et al., 2010a, 2011). Raw milk standardized with skim milk powder at 3.5% (wt/vol; Atilatti, Itatiba, Brazil) was submitted to heat treatment (95°C for 15 min), cooled to 45°C, and inoculated simultaneously with 1% (vol/vol) *Strep. thermophilus* TA040, 1% (vol/vol) *Lb. delbrueckii* ssp. *bulgaricus* LB340, 2% (vol/vol) *B. longum* BL05, and 2% (vol/vol) *Lb. acidophilus* La14. The inoculated milk was subsequently submitted to fermentation at 45°C, with the pH monitored until it reached values of 4.6 ± 0.05 . The mixture was then cooled to 10°C and the gel was broken by manually stirring it for 10 min. Because stirring is the last step that incorporates oxygen during yogurt processing, the glucose oxidase was added during stirring so more consistent results could be obtained. Powdered glucose oxidase (Glucamax CO, Prozyn, São Paulo, Brazil; contained catalase in the formulation) previously dissolved in 100 mL of deionized water was added to yield concentrations of 0, 250, 500, 750, and 1,000 mg/kg (Y_0 , Y_{250} , Y_{500} , Y_{750} , and Y_{1000} , respectively), based on preliminary tests and economic considerations regarding the addition of the enzyme.

Finally, the probiotic yogurts were packaged in 100-mL polypropylene pots (permeability of 0.20 cm³ of O₂ per pot·day, Dixie Toga, São Paulo, Brazil) and stored (3–5°C) for 30 d. Physicochemical and microbiological analyses were conducted at 1, 15, and 30 d.

Microbiological Analysis

The *Strep. thermophilus* TA040 count was quantified using M17 agar (Oxoid) following incubation under aerobiosis at 37°C for 48 h, whereas the *Lb. bulgaricus*

LB 340 count was quantified on de Man, Rogosa, and Sharpe agar (Oxoid) at pH 5.2 (adjusted with glacial acetic acid) and under anaerobiosis at 45°C for 72 h (Rybka and Kailasapathy, 1996). The *B. longum* BL05 count was quantified using lithium chloride-sodium propionate agar containing 0.5 g/L of LiCl and 0.75 g/L of NaCl, heat sterilized, (Sigma), and the plates were incubated under anaerobiosis at 37°C for 72 h (Zacarchenco and Roig, 2004). *Lactobacillus acidophilus* counts were carried out on de Man, Rogosa, and Sharpe agar supplemented with 0.15% (wt/vol) bile salts (Oxoid), incubated at 37°C for 72 h under aerobic conditions (Mortazavian et al., 2007). All the culture media were tested previously to ensure their selectivity for the specific microorganisms. Microbiological analyses were carried out in triplicate.

Physicochemical Analysis

The probiotic stirred yogurts were submitted to the following analyses at 1, 15, and 30 d of refrigerated storage using methodologies published elsewhere: pH (Marshall, 1993); proteolytic activity using the α -phthaldialdehyde (OPA) method (Church et al., 1983); oxygen dissolved (Cruz et al., 2010a); glucose, lactic acid, and acetic acid contents (Donkor et al., 2005); and diacetyl and acetaldehyde (Concurso et al., 2008) contents. The physicochemical analyses were carried out in triplicate.

Statistical Analyses

The experiment was repeated 3 times. Two one-way ANOVA were carried out: the first used sample and the second used storage time as the source of variation. These calculations were followed by Tukey's test; $P < 0.05$ was considered significant. All analyses were carried out using Statistica 7.1 software (StatSoft, Tulsa, OK).

RESULTS AND DISCUSSION

Postacidification, Oxygen Levels, and Proteolysis

Table 1 shows the values for pH, dissolved oxygen, and proteolysis in the probiotic yogurts supplemented with increasing concentrations of glucose oxidase. Overall, the concentration of glucose oxidase throughout the storage time ($P < 0.05$) was found to affect the characteristics of the finished product compared with the control yogurt (Y_0).

The addition of greater amounts of enzyme to the yogurts (Y_{750} and Y_{1000}) induced more extensive post-acidification, as expressed by the lower pH values (4.12 and 4.07 at 30 d, respectively), and a sharp increase in the amount of dissolved oxygen (6.79 and 7.12 g/L at 30 d, respectively), the latter being comparable to that of the control yogurt (7.74 g/L) and intermediate levels of proteolysis (absorbance of 0.678 and 0.698, respectively) during storage. On the other hand, the

Table 1. pH, oxygen content, and proteolysis in probiotic yogurts with increased glucose oxidase concentrations during refrigerated storage¹

Yogurt ²	Storage time (d)	pH	Oxygen (mg/L)	Proteolysis (A_{340}) ³
Y_0	1	4.56 ^{a,A}	0.23 ^{a,A}	0.234 ^{a,A}
Y_{250}	1	4.58 ^{a,A}	0.25 ^{a,A}	0.235 ^{a,A}
Y_{500}	1	4.59 ^{a,A}	0.27 ^{a,A}	0.230 ^{a,A}
Y_{750}	1	4.57 ^{a,A}	0.24 ^{a,A}	0.237 ^{a,A}
Y_{1000}	1	4.55 ^{a,A}	0.26 ^{a,A}	0.233 ^{a,A}
Y_0	15	4.45 ^{a,A}	4.45 ^{a,B}	0.434 ^{c,A}
Y_{250}	15	4.42 ^{a,A}	2.65 ^{b,B}	0.457 ^{ab,B}
Y_{500}	15	4.32 ^{b,B}	2.86 ^{b,B}	0.463 ^{a,B}
Y_{750}	15	4.30 ^{b,B}	1.98 ^{c,B}	0.456 ^{ab,B}
Y_{1000}	15	4.25 ^{c,B}	1.92 ^{c,B}	0.451 ^{b,B}
Y_0	30	4.31 ^{a,C}	7.74 ^{a,C}	0.589 ^{c,C}
Y_{250}	30	4.29 ^{b,C}	4.98 ^{c,C}	0.626 ^{b,C}
Y_{500}	30	4.16 ^{b,C}	4.12 ^{c,C}	0.644 ^{a,C}
Y_{750}	30	4.12 ^{b,C}	6.79 ^{b,C}	0.678 ^{ab,C}
Y_{1000}	30	4.07 ^{c,C}	7.12 ^{ab,C}	0.698 ^{a,C}

^{a-c}Different lowercase letters in the same column indicate presence of statistical difference ($P < 0.05$) among treatments (yogurts).

^{A-C}Different uppercase letters in the same column indicate presence of statistical difference ($P < 0.05$) across storage days.

¹Analyses were performed in duplicate.

² Y_0 , Y_{250} , Y_{500} , Y_{750} , and Y_{1000} = probiotics produced with the addition of 0, 250, 500, 750, and 1,000 mg/kg, respectively, of glucose oxidase.

³Absorbance at 340 nm.

addition of intermediate levels (Y_{250} and Y_{500}) led to a lesser increase in the levels of dissolved oxygen (4.98 and 4.12 g/L at 30 d, respectively), and intermediate pH (4.29 and 4.16, respectively) and proteolysis values (absorbance of 0.626 and 0.644, respectively) throughout the storage period. The results suggest that if, on the one hand, the naturally low pH values of yogurt enhance the importance of an additional amount of glucose oxidase, then, on the other hand, supplementation with excessive amounts may be ineffective because no glucose is available (glucose is the substrate necessary for activating the glucose oxidase). In this way, intermediate amounts of the enzyme yield favorable results throughout the shelf life; that is, low values of dissolved oxygen and milder postacidification and proteolysis, with a significant effect on the economic aspects of production.

Probiotic bacteria, particularly bifidobacteria, are sensitive to low pH values and demonstrate variable proteolytic activity depending on the specific strain (Shihata and Shah, 2000; Jayamane and Adams, 2009) as a function of the presence of the FoF1-ATPase enzyme (van de Guchte et al., 2002). However, the decrease in pH during refrigerated storage is one of the main factors responsible for the poor viability of this microbial group throughout the shelf life of probiotic yogurts (Shah, 2000; Granato et al., 2010b). Strategies used to minimize the effect of the acid stress during probiotic yogurt processing include adequate selection of strains (Godward et al., 2000) and adaptation of the probiotic strain to sublethal stress conditions. Other artifices include the use of selected or genetically modified strains of *Lb. bulgaricus* with slower metabolic rates, which results in a slower decline in pH and decreased proteolytic activity (Ongol et al., 2007), and microencapsulation (Burgain et al., 2011). However, excluding *Lb. bulgaricus* from the yogurt-making process, which would seem to be the most practical and economic alternative, would have regulatory implications because it would decharacterize the product. According to Brazilian regulations, a conventional yogurt must contain at least 7 log cfu/g of viable lactic bacteria cells (Brasil, 2007).

Exposure to oxygen is detrimental for the metabolism and survival of *Bifidobacterium* species as they present anaerobic metabolism. The oxidative damage is related to the production of reactive oxygen species, and the bacteria tolerance to this stress is associated to presence of some enzymes and also changes in morphology and components on the cell surface (Ruiz et al., 2011) and this effect is strain-dependent. An increase in the growth of *B. longum* during the lag phase and some morphological modifications as well as changes in the fatty acid profile have already been reported (Ahn

et al., 2001), whereas, for *B. animalis* ssp. *lactis* Bb12, changes in the surface hydrophobicity and an increase in protein content in the presence of increased oxygen concentration was observed by Shakirova et al. (2010). Other factors to be considered are that aerobic conditions decrease the production of exopolysaccharides in some bifidobacteria strains, such as *B. longum* (Ninomiya et al., 2009), and this can be considered negative, as exopolysaccharides are able to increase viscosity and confer a desirable texture to the fermented product (Salazar et al., 2009).

Minimizing oxidative stress in the processing of probiotic yogurts has been extensively researched for maintenance of viable probiotic bacteria counts, with several alternative technologies being proposed. Recently, it was reported that addition of nitrogen during yogurt processing simultaneously with the addition of the cultures and subsequent fermentation at 37°C could result in a decrease in the amount of dissolved oxygen in the product before the production of acids (Horiuchi et al., 2009).

Our findings presented results that are comparable to previous technological solutions, such as the addition of ascorbic acid or other ingredients that are used to minimize the oxidative stress caused by oxygen (Dave and Shah, 1997, 1998), milk electroreduction (Bolduc et al., 2006), the use of polystyrene laminated to high-gas-barrier material (Miller et al., 2002), active packaging systems (Miller et al., 2003), microencapsulation (Talwalkar and Kailasapathy, 2004), the use of plastic packaging systems with different polarities and crystallinities (Jasson et al., 2001), and the use of Oxyrase (Oxyrase Inc., Mansfield, OH), which reduces fractions of bacterial cell membranes (Ordóñez et al., 2000). The supplementation of probiotic yogurts with intermediate glucose oxidase concentrations decreased the oxygen level and provided intermediate proteolysis values during yogurt storage. Although it is possible to induce adaptations to oxidative stress in probiotic bacteria (Talwalkar and Kailasapathy, 2004), it is a time-consuming task, whereas the addition of glucose oxidase during yogurt processing is a simple option, available to small and medium-sized dairy processors.

Microbial Counts

Table 2 shows the viable counts of *Strep. thermophilus*, *Lb. bulgaricus*, *B. longum*, and *Lb. acidophilus* in probiotic yogurts supplemented with increasing quantities of glucose oxidase. Immediately upon completion of fermentation, counts of 9.38, 8.53, 8.01, and 8.6 log cfu/mL were recorded for these microorganisms, respectively, suggesting that this step did not have a negative effect on the growth of probiotic and starter microor-

Table 2. Microbiological viable count of probiotic and starter bacteria in probiotic yogurts with increased glucose oxidase concentrations during the refrigerated storage¹

Yogurt ²	Storage time (d)	Bacterial count (cfu/mL)			
		<i>Streptococcus thermophilus</i>	<i>Lactobacillus bulgaricus</i>	<i>Bifidobacterium longum</i>	<i>Lactobacillus acidophilus</i>
Y ₀	1	9.34 ^{a,A}	8.78 ^{a,A}	7.52 ^{a,A}	8.45 ^{a,A}
Y ₂₅₀	1	9.31 ^{a,A}	8.65 ^{a,A}	7.77 ^{a,A}	8.32 ^{a,A}
Y ₅₀₀	1	9.34 ^{a,A}	8.14 ^{a,A}	7.81 ^{a,A}	8.38 ^{a,A}
Y ₇₅₀	1	9.30 ^{a,A}	8.34 ^{a,A}	7.43 ^{a,A}	8.35 ^{a,A}
Y ₁₀₀₀	1	9.35 ^{a,A}	8.67 ^{a,A}	7.61 ^{a,A}	8.45 ^{a,A}
Y ₀	15	9.29 ^{a,A}	8.45 ^{a,A}	7.12 ^{c,B}	8.45 ^{a,B}
Y ₂₅₀	15	9.04 ^{a,A}	8.23 ^{a,A}	8.27 ^{a,B}	7.23 ^{b,B}
Y ₅₀₀	15	9.24 ^{a,A}	8.32 ^{a,A}	8.23 ^{a,B}	8.62 ^{a,B}
Y ₇₅₀	15	9.87 ^{a,A}	8.28 ^{a,A}	7.34 ^{bc,B}	8.55 ^{a,B}
Y ₁₀₀₀	15	9.76 ^{a,A}	8.38 ^{a,A}	7.51 ^{b,B}	7.38 ^{b,B}
Y ₀	30	9.38 ^{a,A}	8.34 ^{a,A}	6.41 ^{c,C}	6.34 ^{c,C}
Y ₂₅₀	30	9.50 ^{a,A}	8.67 ^{a,A}	7.93 ^{a,C}	8.22 ^{a,C}
Y ₅₀₀	30	9.65 ^{a,A}	8.88 ^{a,A}	8.06 ^{a,C}	8.12 ^{a,C}
Y ₇₅₀	30	9.54 ^{a,A}	8.75 ^{a,A}	6.88 ^{c,C}	6.65 ^{b,C}
Y ₁₀₀₀	30	9.46 ^{a,A}	8.46 ^{a,A}	6.72 ^{b,C}	6.66 ^{b,C}

^{a-c}Different lowercase letters in the same column indicate presence of statistical difference ($P < 0.05$) among treatments (yogurts).

^{A-C}Different uppercase letters in the same column indicate presence of statistical difference ($P < 0.05$) across storage days.

¹Analyses were performed in duplicate.

²Y₀, Y₂₅₀, Y₅₀₀, Y₇₅₀, and Y₁₀₀₀ = probiotics produced with the addition of 0, 250, 500, 750, and 1,000 mg/kg, respectively, of glucose oxidase.

ganisms. *Streptococcus thermophilus* and *Lb. bulgaricus* counts varied from 9.04 (Y₂₅₀, d 15) to 9.87 (Y₇₅₀, d 15) and from 8.14 (Y₅₀₀, d 1) to 8.88 (Y₅₀₀, d 30) log cfu/mL throughout the period investigated, suggesting that the viability of the starter cultures was not influenced by the addition of the enzyme.

However, with regard to the counts of probiotic microorganisms, both the concentration of glucose oxidase and storage time ($P < 0.05$) were observed to have an effect on the viable counts of microorganisms compared with the control yogurt (Y₀). Yogurts with high concentrations of glucose oxidase (Y₇₅₀ and Y₁₀₀₀) showed a more rapid decrease in the numbers of both species: *B. longum* declined from 7.43 and 7.61 to 6.88 and 6.72 in Y₇₅₀ and Y₁₀₀₀, respectively, whereas the *Lb. acidophilus* counts declined from 8.35 and 8.45 to 6.65 and 6.66 log cfu/mL. In both cases, the reductions in the number of viable cells were comparable to the decreases observed in the control yogurt, which presented counts of 6.41 and 6.34 log cfu/mL, respectively, at the end of the storage period. These results coincided with the increase in oxygen content in the yogurt and reinforce the difficulty of minimizing oxidative stress in probiotic yogurts.

It is noteworthy that although glucose oxidase can produce hydrogen peroxide, which would cause an inhibitory effect on probiotic bacteria, the commercial enzyme product used in this study contained catalase in its formulation. This minimized any negative effect, as catalase consumes hydrogen peroxide produced during glucose oxidase action. However, it also produces

oxygen, which can be incorporated throughout the refrigerated storage, and therefore may be considered a disadvantage. However, the amount produced did not seem comparable to the oxygen that permeates through the packaging during the storage. The results suggest that the addition of glucose oxidase had an overall positive effect in terms of maintaining or reducing the increase of oxygen dissolved in the probiotic yogurts compared with the control yogurt and consequently created favorable conditions for the development of probiotic microorganisms, which are intrinsically anaerobic or microaerophilic. Addition of glucose oxidase is a biotechnological option and this approach is in agreement with the tendency of the modern food industry where there is negative consensus about the inclusion of chemical compounds in foods (Behrens et al., 2010; Shim et al., 2011). Nonetheless, high levels of supplementation are not necessary given the lack of substrate available in the system. Further studies should include a consumer test aimed to check the optimal concentration at which glucose oxidase can be added to the yogurt with respect to consumer perception.

Production of Lactic Acid, Acetic Acid, Diacetyl, and Acetaldehyde

Table 3 shows the values for lactic acid, acetic acid, diacetyl, and acetaldehyde in the probiotic yogurts supplemented with increasing quantities of glucose oxidase. Overall, a positive influence of glucose oxidase and storage time ($P < 0.05$) was observed compared with the

Table 3. Production of lactic acid, acetic acid, diacetyl, and acetaldehyde in probiotic yogurts with increased glucose oxidase concentrations during the refrigerated storage (glucose was not detected in any yogurt throughout the study)¹

Yogurt ²	Storage time (d)	Lactic acid (mg/mL)	Acetic acid (mg/mL)	Diacetyl (µg/mL)	Acetaldehyde (µg/mL)
Y ₀	1	5.43 ^{a,A}	0.75 ^{a,A}	16.7 ^{c,A}	25.3 ^{c,A}
Y ₂₅₀	1	5.57 ^{a,A}	0.57 ^{a,A}	19.4 ^{b,A}	59.4 ^{b,A}
Y ₅₀₀	1	5.26 ^{a,A}	0.82 ^{a,A}	17.4 ^{bc,A}	98.0 ^{a,A}
Y ₇₅₀	1	5.37 ^{a,A}	0.79 ^{a,A}	23.6 ^{b,A}	51.9 ^{b,A}
Y ₁₀₀₀	1	5.18 ^{a,A}	0.84 ^{a,A}	41.1 ^{a,A}	58.7 ^{b,A}
Y ₀	15	4.72 ^{b,B}	0.57 ^{c,B}	14.4 ^{b,B}	69.6 ^{a,B}
Y ₂₅₀	15	5.22 ^{b,A}	0.79 ^{b,B}	12.8 ^{c,B}	25.9 ^{c,B}
Y ₅₀₀	15	5.50 ^{a,B}	0.75 ^{b,B}	17.5 ^{ac,B}	71.6 ^{a,B}
Y ₇₅₀	15	5.62 ^{a,B}	0.83 ^{a,B}	17.4 ^{a,B}	44.4 ^{b,B}
Y ₁₀₀₀	15	5.95 ^{a,B}	0.86 ^{a,B}	13.9 ^{bc,B}	28.2 ^{c,B}
Y ₀	30	6.42 ^{b,C}	0.77 ^{a,B}	88.3 ^{a,C}	11.3 ^{b,C}
Y ₂₅₀	30	6.54 ^{b,C}	0.52 ^{c,B}	97.2 ^{a,C}	23.8 ^{a,C}
Y ₅₀₀	30	6.77 ^{a,C}	0.53 ^{c,B}	88.1 ^{a,C}	22.2 ^{a,C}
Y ₇₅₀	30	7.08 ^{a,C}	0.62 ^{b,B}	30.9 ^{c,C}	24.2 ^{a,C}
Y ₁₀₀₀	30	7.80 ^{a,C}	0.66 ^{b,B}	56.4 ^{b,C}	23.3 ^{a,C}

^{a-c}Different lowercase letters in the same column indicate presence of statistical difference ($P < 0.05$) among treatments (yogurts).

^{A-C}Different uppercase letters in the same column indicate presence of statistical difference ($P < 0.05$) across storage days.

¹Analyses were performed in duplicate.

²Y₀, Y₂₅₀, Y₅₀₀, Y₇₅₀, and Y₁₀₀₀ = probiotics produced with the addition of 0, 250, 500, 750, and 1,000 mg/kg, respectively, of glucose oxidase.

control yogurt. Glucose was not detected in any of the yogurts tested, suggesting that it was entirely consumed by the enzyme from the beginning of the shelf life (d 1). However, the consumption of glucose by probiotic or starter microorganisms cannot be discounted. Further studies are needed to clarify this effect.

Our results support that increased levels of glucose oxidase enhanced the metabolism of the lactic and probiotic cultures that readily benefited from the smaller quantities of dissolved oxygen due to glucose oxidase action. Obviously, in the course of the refrigerated storage, the enzyme loses its activity and the dissolved oxygen levels may increase. This effect may lower the counts of bifidobacteria and consequently decrease the acetic acid production. With regard to the characteristic aroma compounds of yogurt; that is, diacetyl and acetaldehyde, it is important that the addition of glucose oxidase does not negatively affect the typical flavor of the product. The results obtained in this study are promising in that, throughout the storage period evaluated, the yogurts with glucose oxidase were found to produce greater amounts ($P < 0.05$) of these 2 compounds compared with the control yogurt. As for diacetyl, the values determined in the experimental yogurts varied from 19.4 (Y₂₅₀, d 1) to 97.2 (Y₂₅₀, d 30) mg/L, with the control yogurt (Y₀) showing variation from 8.29 to 16.7 mg/mL, respectively.

Greater production of acetaldehyde ($P < 0.05$) was also observed, which is extremely favorable because this compound is mainly responsible for the typical aroma of yogurt (Cheng, 2010). Maximum production was observed after 15 d of refrigerated storage, 69.6 (Y₀) to

71.6 (Y₅₀₀) µg/mL, respectively, declining to 23.3 (Y₁₀₀) and 13.8 (Y₂₅₀) at the end of the storage period. The results are coherent, because overall, probiotic bacteria do not produce aroma compounds markedly and, in general, probiotic yogurts and fermented milks are low in flavor due to the low activity of the enzyme threonine aldolase, which catalyzes the formation of acetaldehyde from the substrate, threonine (Gardini et al., 1996). The decrease in acetaldehyde levels at the end of the storage time can be related to the presence of enzyme alcohol dehydrogenase, which metabolizes acetaldehyde in ethanol. This enzyme has been detected in probiotic bacteria such as *Lb. acidophilus* (Marshall and Cole, 1983), and it can explain the mild flavor present in probiotic yogurts, as acetaldehyde has a lower threshold compared with ethanol.

Baranowska (2006) reported that levels of lactose and glucose present in milk did not influence the production of diacetyl and acetaldehyde in yogurt, but did influence supplementation of the yogurt with the precursors of aroma compounds. A recent study (Pinto et al., 2009) confirmed this finding when investigating the behaviors of diacetyl and acetaldehyde in yogurts processed with single and joint cultures throughout shelf life. The authors showed that the amount of each compound produced depended on the cultures used and the interactions between them. In general, the production of diacetyl was greater in yogurts fermented by *Strep. thermophilus* on its own, whereas the production of acetaldehyde was greater in yogurts fermented with *Lb. bulgaricus*. Ott et al. (2000) report that the difference in production of flavor compounds in yogurt

may be related to the intensity of the acidity of the products.

Recently, Martin et al. (2011) reported on the influence of the oxidoreduction potential value on the production of aroma compounds, as it can modify the metabolic pathways of the yogurt bacteria. Oxidative conditions contribute to the stability of the acetaldehyde during the yogurt storage, whereas reducing conditions provide the opposite effect.

Our approach is relevant because we observed no negative effect of glucose oxidase supplementation on production of aroma compounds in yogurt. This is crucial for its acceptance by the consumers as probiotic and conventional yogurts should be similar for consumers. In this context, adequate production of acetaldehyde and diacetyl, which are the compounds related to the typical flavor of the yogurt (Cheng, 2010), is essential.

CONCLUSIONS

The results of this study suggest that addition of glucose oxidase is a potential option for maintaining the survival of probiotic bacteria in yogurts. Yogurts with added glucose oxidase presented a lesser increase in the concentration of dissolved oxygen, along with a smaller decrease in the counts of *B. longum* and *Lb. acidophilus*, were observed. In addition, the typical yogurt aroma compounds, acetaldehyde and diacetyl, were found to be produced in adequate amounts, together with the formation of moderate amounts of organic acids, particularly lactic acid and acetic acid. Supplementation with high levels of glucose oxidase was not an adequate alternative because of the lack of available substrate necessary for its action, and higher levels of glucose oxidase enzyme resulted in higher concentrations of oxygen dissolved in the product, which, in turn, led to lower counts of lactic and probiotic bacteria. Future studies should assess the performance of probiotic yogurts supplemented with glucose oxidase and packaged in systems with different oxygen permeabilities. Furthermore, affective tests based on consumer perception should be performed to evaluate the changes in the sensory characteristics of the probiotic yogurts caused by the addition of increasing quantities of glucose oxidase.

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